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Cold Storage of *Culex pipiens* in the Absence of Diapause

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ABSTRACT A major expenditure in vector biology laboratories is the rearing of mosquitoes. Most mosquito colonies require substantial effort to maintain, including frequent bloodmeals for optimal performance. Successful cryopreservation of mosquitoes continues to be elusive. Although using diapause as a storage mechanism is an option for mosquito preservation, several obstacles include the lack of a well-characterized diapause or the inability of some species to enter diapause. Thus, other options for preservation are needed. To address this issue, we investigated the use of long-term low-temperature storage in the absence of diapause for adults of the northern house mosquito, *Culex pipiens* L. Our results indicate that although male longevity is not substantially increased by cold storage, female longevity is dramatically increased by storage at lower temperatures. When mated before storage, females remain reproductively viable after at least 10 wk of storage, although at reduced levels. These results indicate that cold storage without diapause induction is a viable option for colony maintenance in vector biology laboratories.

KEY WORDS *Culex pipiens*, northern house mosquito, cold storage, fecundity

A major expense of many entomology laboratories, both in terms of time and resources expended, is the maintenance of laboratory cultures of the species of interest. This maintenance is especially true for disease vectors such as mosquitoes that must be regularly fed bloodmeals to generate subsequent generations. As growing numbers of mosquito genomes are characterized, and as molecular techniques give rise to ever increasing numbers of mosquito strains, the operational costs of mosquito rearing promise to also steadily increase. In addition to the operational costs of continuous colony maintenance, there can be biological costs. Rearing insects for multiple generations under laboratory conditions has long been known to cause genetic drift (Mackauer 1972, Chambers 1977), ultimately leading to deleterious effects such as the gradual loss of fecundity (Franz et al. 1996) or other key characteristics (Mackauer 1976, Zheng et al. 1993, Goto et al. 2006).

Alternatives to continuous culture do exist. Cryopreservation has been successful for several insect species, including the house fly, *Musca domestica* L.; the screwworm, *Cochliomyia hominivorax* (Coquerel); a biting midge; and several species of tephritid fruit flies (Leopold 2007). However, the technique must be adapted for each species, and some insects, including mosquitoes, have morphological characteristics that thus far seem to be insurmountable (Valencia et al. 1996a,b) and would require the development of novel approaches. Diapause also can be used as a method of storage between generations (Denlinger 2008) but may be difficult to implement as well. Limitations to using diapause for insect storage include the lack of or poorly understood diapause in many species (including several important tropical mosquitoes), and the fact that the requirements of diapause initiation and termination must be characterized for each species. Confounding factors include genetic drift, which can lead to the loss of the ability to diapause (Goto et al. 2006), and the development of nondiapausing transgenic strains.

Hence, the development of alternative storage techniques would be beneficial to the entomological community. The experiments presented here tested the ability of the northern house mosquito, *Culex pipiens* L., to be stored under low-temperature conditions without the induction of diapause as well as how the extended exposure to cold affects the fecundity of the stored insects and their subsequent generations.

Materials and Methods

Insects. An anautogenous colony of the Buckeye strain of *Cx. pipiens* (Robich et al. 2007) was used throughout this study. Adults were maintained at 25°C, 75% humidity, and a photoperiod of 15:9 (L:D) h and were provided honey-soaked sponges. Adults were allowed to blood feed weekly on an anesthetized

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rat. Eggs, larvae, and pupae were reared at 25°C and a photoperiod of 15:9 (L:D) h in shallow pans of reverse osmosis-treated water. Larvae were fed pulverized fish food flakes (TetraMin, Inc. Blacksburg, VA) until pupation. This treatment prevented the initiation of diapause that in this species is brought about by rearing at 18°C and a short-day photoperiod of 9:15 (L:D) h (Rinehart et al. 2006).

Temperature Treatments. When destined for experiments, pupae were removed from larval pans and placed in emergence cups which were then placed in a clean cage (45 by 45 by 45 cm). Emergence was allowed to continue for 3 d, after which the emergence cups were removed from the cage. The adults were maintained in this cage for 1 wk after cup removal at 25°C and a photoperiod of 15:9 (L:D) h with access to honey-soaked sponges as well as water-soaked sponges, to allow for mating to occur. After 1 wk, adult mosquitoes (7–10 d old) were aspirated from the cage in sets of 15 males or females, briefly anesthetized with carbon dioxide, and placed in 473-ml plastic cages (16-oz. "deli cups") topped with nylon window screen. Three of these cages were then placed inside 25-cm-diameter, 10-cm-high clear plastic containers containing cups of saturated sodium chloride solution to maintain 75% humidity inside the container. Because this species is unable to absorb atmospheric water (Benoit and Denlinger 2007), a 4- by 6-cm water-soaked sponge was placed on top of each deli cup cage. The plastic containers (each containing three cages) were then placed in either model I25L (Conviron, Winnipeg, MB, Canada) or model I30BLL (Percival Scientific, Perry, IA) reach-in chambers programmed to maintain a photoperiod of 15:9 (L:D) h with one of four constant temperature treatments (6, 12, 18, or 25°C). This process was repeated three times for each sex.

The cages were removed from the environmental chambers weekly; water sponges were washed and replenished; and survivorship, defined as the ability of a mosquito to right itself, was assessed. This design (one clear plastic container containing three deli cup cages of 15 adults) was repeated three times for each treatment, with each replicate using adult mosquitoes reared from a different blood feeding.

Assessment of Fecundity. To assess the fecundity of stored females, separate sets of cages were established as described previously and were then maintained until the calculated LT_{50} value of the respective treatment temperature had been reached. To circumvent poor blood-feeding rates immediately after storage, mosquitoes were acclimated at 25°C for an additional week before being offered a bloodmeal. To ameliorate mortality rates during this week at 25°C, honey-soaked sponges were added to the top of the cages in addition to the water-soaked sponges. At the end of the week, mosquitoes were transferred to a cage (20 by 20 by 20 cm) for blood feeding and oviposition. Egg rafts were removed from the cages and photographed to assist in counting the number of eggs per raft by using a Moticam 2000 imaging system (Motic, Inc., Richmond, BC, Canada) attached to an Olympus (Center Valley, PA) SZH10 stereomicroscope. Individual egg rafts were transferred to separate wells of a six-well plate to assess hatching rates, with the number of larvae per well determined by photography and image analysis. After hatching, larvae were transferred to 473-ml deli cups, fed pulverized fish food daily, and the number of pupae produced by each cup was tallied to determine larval survival rates. For the 6 and 12°C storage groups, pupae were transferred to separate cage (45 by 45 by 45 cm) to determine the fecundity of the F1 and F2 generations. As a reference, 12 egg rafts were analyzed from a colony cage containing females encountering their first bloodmeal to determine the average fecundity of this strain of *Cx. pipiens* reared under our conditions.

Statistical Analysis. Female median lethal time (LT_{50}) and time to 75% mortality (LT_{75}) values for each of the three replicates were calculated for each treatment group by using Kaplan–Meier survival analysis. Differences between the groups were analyzed by one-way analysis of variance (ANOVA) and Holm–Sidak post hoc analysis. Male survival curves were not calculated. Fecundity data were analyzed by one-way ANOVA and Bonferroni post hoc analysis.

Results

Cold Storage and Survival. Both male (Fig. 1) and female (Fig. 2) adult nondiapausing *Cx. pipiens* exhibited increased longevity with decreased incubation temperatures. The effect was less pronounced with adult males, with those reared at 25°C reaching 100% mortality at 2 wk, and those stored at 6°C expiring by 4 wk of storage (Fig. 1). In addition, rather than a progressive dose response to temperature as seen for female mosquitoes, male survival curves exhibit grouping, with the 12 and 6°C curves nearly overlapping.

The effect of storage temperatures on adult survival was much more pronounced for female mosquitoes (Fig. 2). Whereas all individuals died after being held for 28 d at 25°C, 100% mortality was not attained until after 49 d at 18°C, 56 d at 12°C, and 16 wk at 6°C. The calculated LT_{50} values demonstrate this trend as well, with a dramatic increase of LT_{50} values from 18.7 ± 2.33 at 25°C to 25.7 ± 2.33 at 18°C, 44.3 ± 2.33 at 12°C, and 70.0 ± 4.04 d for females stored at 6°C (Fig. 3). One-way ANOVA confirmed significant variation in female LT_{50} values as well ($F_{3,11} = 64.44$; $P < 0.001$) with Holm–Sidak post hoc analysis indicating statistically significant differences among all treatment groups at the $P = 0.05$ level. The calculated LT_{75} values showed a similar trend, with one-way ANOVA, indicating significant variation ($F_{3,11} = 85.87$; $P < 0.001$) and Holm–Sidak post hoc analysis demonstrating significant differences among all treatment groups. The relatively small differences between calculated LT_{50} values and LT_{75} values (Fig. 3) are consistent with the steepness of the survivorship curves (Fig. 2).

Cold Storage and Fecundity. Cold storage of adult females resulted in a statistically significant reduction in fecundity at the calculated LT_{50} of the 6 and 12°C

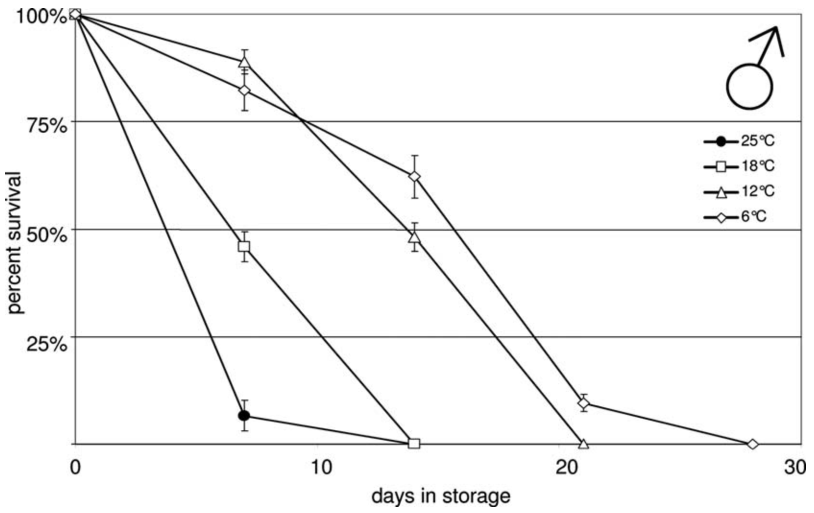

Fig. 1. Percentage of survival (mean ± SE) of *Cx. pipiens* adult males stored at various temperatures.

temperature treatment groups, when measured as the number of eggs produced per female ($F_{8,93}$ = 19.66; $P < 0.0001$) compared with the normal egg production rates of the colony. Post hoc analysis revealed that neither 14 d at 25°C nor 23 d at 18°C had a statistically significant effect on egg raft size. However, 36 d at 12°C did significantly reduce the size of the egg raft by 48% of those produced by colony mosquitoes and 65 d at 6°C reduced egg raft size by 51% (Fig. 4).

Interestingly, egg raft sizes for the 12 and 6°C treatment groups did not fully recover in the F1 generation. The F1 generation of the 12°C treatment group showed a 9% reduction in egg raft size, whereas the 6°C treatment group showed a 21% reduction as compared with controls. This decline in egg production was statistically significant for only the 6°C treatment group. In both groups, egg production returned to normal levels by the F2 generation. Other parameters of fecundity were not affected by cold storage. Neither larval hatching rates ($F_{8,93}$ = 1.52; $P < 0.1599$) nor percentage of larvae developing to the pupal stage ($F_{8,93}$ = 0.10; $P < 0.9990$) exhibited statistically different values among the treatment groups (data not shown).

Discussion

Our results demonstrate that adult *Cx. pipiens* can be successfully stored at reduced temperatures, even when diapause was not initiated. Increasing the longevity of males was modestly successful; survival curves were dose responsive to incubation temperature, but no adult males survived past 4 wk. Increased longevity of females was much more pronounced. Although incubating females at 18°C led to only approximately a 1-wk increase in LT_{50} values, incubating

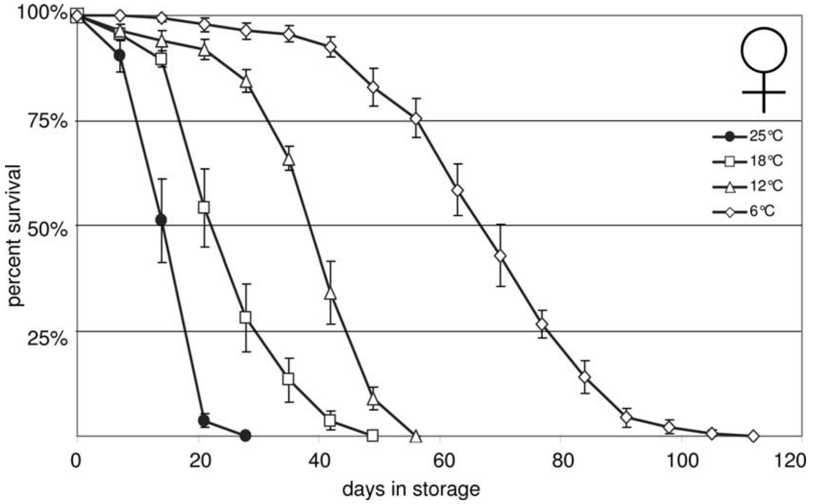

Fig. 2. Percentage of survival (mean ± SE) of *Cx. pipiens* adult females stored at various temperatures.

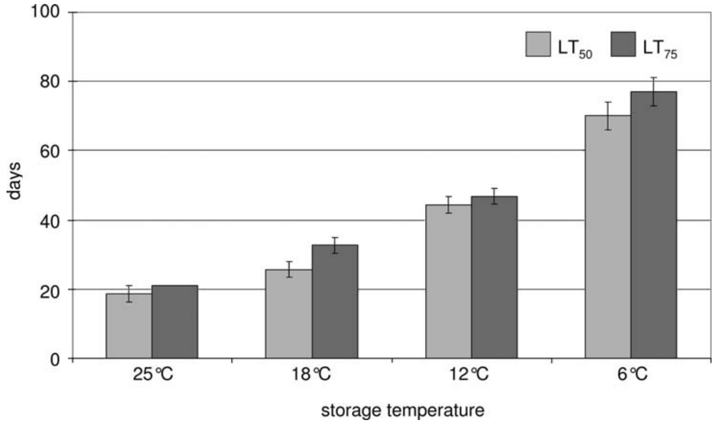

Fig. 3. Calculated LT_{50} and LT_{75} values (mean ± SE) for female adult *Cx. pipiens* stored at various temperatures. All LT_{50} values and LT_{75} values are statistically different from one another.

at 12°C caused a 2.5-fold increase in LT_{50} values (from 18 d to 44 d), and incubating at 6°C caused a nearly four-fold increase (to 70 d). This sexual dimorphism of survival is similar to what is seen during diapause in this species. Diapause is exclusively a female trait, as males are rarely seen at overwintering sites (Spielman 1964). However, the longevity of diapausing *Cx. pipiens* is, in general, far greater than what we observed in our nondiapausing insects. Field observations indicate that *Cx. pipiens* adults seek overwintering sites in late August and early October (Spielman and Wong 1973) where they remain until March or April (Minar and Ryba 1971, Onyeka and Boreham 1987). The difference in longevity between diapausing and cold-stored mosquitoes is probably due to differences in nutritional reserves because a key component of diapause in many insects, including *Cx. pipiens,* is a preparatory phase that includes increased feeding and a subsequent increase in fat reserves (Mitchell 1983, Mitchell and Briegel 1989, Bowen 1992). Whether cold storage can be improved by sugar feeding remains to be elucidated.

Although cold-stored females did live longer, increased longevity did not come without cost. When assessed at the LT_{50} females stored at 12 and 6°C exhibited decreased egg production compared with their counterparts reared under normal conditions. Conversely, incubation at 18°C had no such affect. Interestingly, the reduction in egg production was not limited to the stored generation. Although the egg production of the F1 generation resulting from females stored at 12°C was not statistically different from controls, the egg production of the F1 generation resulting from females stored at 6°C was significantly lower than their counterparts reared under normal conditions, but still higher than the parental generation. Egg production was not restored to normal levels until the F2 generation. Reduced fecundity in the progeny of insects that have undergone stress, including storage, has been previously documented many insects (Leopold 1998, Pitcher et al. 2002, López and Botto 2005, Chen et al. 2008).

How can a cold storage protocol based on the above observations assist facilities rearing *Cx. pipiens*? When

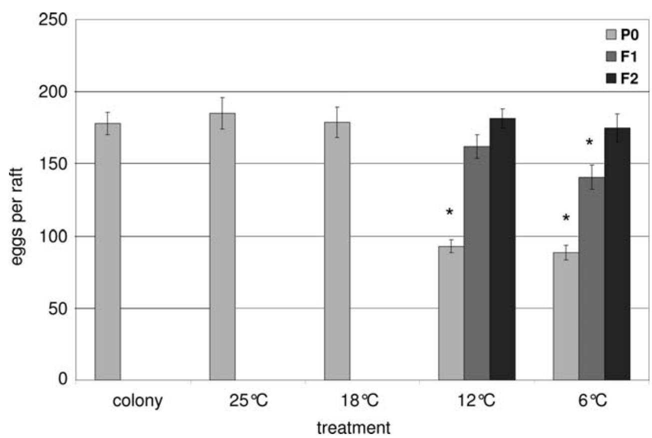

Fig. 4. Effect of cold storage on egg production when measured at the LT_{50} value of the respective storage temperature. P0, parental (stored) generation; F1 and F2, subsequent generations. Asterisk (*) denotes values that are statistically significant from colony mosquitoes.

following a discrete generation protocol, one can expect a generation of mosquitoes every 3 wk (Moll et al. 2008), totaling ≈17 generations a year. In our hands, this represents roughly 8 h of work per generation or ≈140 h per colony each year. Conversely, when storing adult females at 6°C, you can expect a generation of mosquitoes every 15 wk: 1 wk for mating, 10 wk of storage, 1 wk of recovery, and 3 wk to produce the subsequent generation. This results in ≈3.5 generations per year. The work load during storage is minimal, consequently requiring ≈9 h of effort per generation or ≈32 h per colony each year. Hence, incorporating cold storage into the protocol would result in a reduction in cost of over 75%, both in terms of work load and finances, because the expenses incurred during storage are minimal. An alternative interpretation is that a facility could maintain 4 times the number of colonies without substantially increasing maintenance costs when cold storage is used.

How do our other observations affect the implementation of cold storage for *Cx. pipiens* colonies? The sexual dimorphism of survival, although intriguing from a cold physiology perspective, is largely irrelevant to cold storage, because mating is allowed to occur before storage, thereby circumventing this issue. The reduction in fecundity is potentially more troubling, and deserves further study. If the F_1 generation is cold stored, and if the suppression in fecundity is additive, the colony would expire in a few generations. This could be easily rectified by rearing two generations between cold storage cycles, thereby only storing F_2 mosquitoes. This solution would, of course, increase the amount of resources devoted to colony maintenance, although this modified cold storage protocol would still result in a savings of 65% over a discrete generation protocol.

Can these protocols be adapted to other species and strains of mosquitoes, especially those that are more difficult to rear? This remains to be elucidated, although data from other insects are encouraging. For example, a similar set of experiments on the alfalfa leafcutting bee, *Megachile rotundata* (F.), have shown successful storage of nondiapausing individuals at 6°C for this species as well (Yocum et al. 2010). If similar results can be attained for these two diverse species, protocol adaptation for other mosquito species seems likely. Although these results are encouraging, further studies are warranted, including improving storage regimes for *Cx. pipiens* as a model system and adapting these protocols to additional mosquito species.

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References Cited

Benoit, J. B., and D. L. Denlinger. 2007. Suppression of water loss during adult diapause in the northern house mosquito, *Culex pipiens.* J. Exp. Biol. 210: 217–226.

Bowen, M. F. 1992. Patterns of sugar feeding in diapausing and nondiapausing *Culex pipiens* (Diptera: Culicidae) females. J. Med. Entomol. 29: 843–849.

Chambers, D. L. 1977. Quality control in mass rearing. Annu. Rev. Entomol. 22: 289–308.

Chen, W.-L., R. A. Leopold, and M. A. Boetel. 2008. Cold storage of adult *Gonatocerus ashmeadi* (Hymenoptera: Mymaridae) and effects on maternal and progeny fitness. J. Econ. Entomol. 101: 1760–1770.

Denlinger, D. L. 2008. Why study diapause? Entomol. Res. 38: 1–9.

Franz, G., P. Kerremans, P. Rendon, and J. Hendrichs. 1996. Development and application of genetic sexing systems for the Mediterranean fruit fly based on a temperature sensitive lethal, pp. 185–191. *In* B. A. McPheron and G. J. Steck (eds.), Fruit fly pests: a world assessment of their biology and management. St. Lucie Press, Delray Beach, FL.

Goto, S. J., B. Han, and D. L. Denlinger. 2006. A nondiapausing variant of the flesh fly, *Sarcophaga bullata,* that shows arrhythmic adult eclosion and elevated expression of two circadian clock genes, *period* and *timeless.* J. Insect Physiol. 52: 1213–1218.

Leopold, R. A. 2007. Cryopreservation of nonmammalian metazoan systems, pp. 271–298. *In* J. G. Baust and J. M. Baust (eds.), Advances in biopreservation. CRC Taylor and Francis Group, Boca Raton, FL.

Leopold, R. A. 1998. Cold storage of insects for integrated pest management, pp. 235–267. *In* G. J. Hallman and D. L. Denlinger (eds.), Temperature sensitivity in insects and application in integrated pest management. Westview Press, Boulder, CO.

López, S. N., and E. Botto. 2005. Effect of cold storage on some biological parameters of *Eretmocerus corni* and *Encarsia formosa* (Hymenoptera: Aphelinidae). Biol. Control 33: 123–130.

Mackauer, M. 1972. Genetic aspects of insect production. Entomophaga 17: 27–48.

Mackauer, M. 1976. Genetic problems in the production of biological control agents. Annu. Rev. Entomol. 21: 369–385.

Minar, J., and J. Ryba. 1971. Experimental studies on overwintering conditions of mosquitoes. Folia Parasitol. 18: 255–259.

Mitchell, C. J. 1983. Differentiation of host-seeking behavior from blood-feeding behavior in overwintering *Culex pipiens* (Diptera: Culicidae) and observations on gonotrophic dissociation. J. Med. Entomol. 30: 157–163.

Mitchell, C. J., and H. Briegel. 1989. Inability of diapausing *Culex pipiens* (Diptera: Culicidae) to use blood for producing lipid reserves for overwinter survival. J. Med. Entomol. 26: 318–326.

Moll, K., I. Ljungström, H. Perlmann, A. Scherf, and M. Wahlgren (eds.). 2008. Methods in malaria research, 5th ed. MR4. American Type Culture Collection, Manassas, VA.

Onyeka, J.O.A., and P.F.L. Boreham. 1987. Population studies, physiological state and mortality factors of overwintering adult populations of females of *Culex pipiens* L. (Diptera: Culicidae), Bull. Entomol. Res. 77: 99–111.

Pitcher, S. A., M. P. Hoffmann, J. Gardner, M. G. Wright, and T. P. Kuhar. 2002. Cold storage of *Trichogramma ostriniae* reared on *Sitotroga cerealella* eggs. Biocontrol 47: 525–535.

Rinehart, J. P., R. M. Robich, and D. L. Denlinger. 2006. Enhanced cold and desiccation tolerance in diapausing adults of *Culex pipiens,* and a role for Hsp70 in response to cold shock but not as a component of the diapause program. J. Med. Entomol. 43: 713–722.

Robich, R. M., J. P. Rinehart, and D. L. Denlinger. 2007. Diapause-specific gene expression in the northern house mosquito, *Culex pipiens* L., identified by suppressive subtractive hybridization. J. Insect Physiol. 53: 235–245.

Spielman, A. 1964. Studies on autogeny in *Culex pipiens* populations in nature. I. Reproductive isolation between autogenous and anautogenous populations. Am. J. Hyg. 80: 175–183.

Spielman, A., and J. Wong. 1973. Studies on autogeny in natural populations of C*ulex pipi*ens. III. Midsummer preparation for hibernation in anautogenous populations. J. Med. Entomol. 10: 319–324.

Valencia, M.D.P., L. H. Miller, and P. Mazur. 1996a. Permeability of intact and dechorionated eggs of the *Anopheles* mosquito to water vapor and liquid water: a comparison with *Drosophila.* Cryobiology 33: 142–148.

Valencia, M.D.P., L. H. Miller, and P. Mazur. 1996b. Permeabilization of eggs of the malaria mosquito *Anopheles gambiae.* Cryobiology 33: 149–162.

Yocum, G. D., J. P. Rinehart, M. West, and W. P. Kemp. 2010. Interrupted incubation and short-term storage of the alfalfa pollinator *Megachile rotundata* (Hymenoptera: Megachilidae): a potential tool for synchronizing bees with bloom. J. Econ. Entomol. 103: 234–241.

Zheng, Y., K. M. Daane, K. S. Hagen, and T. E. Mittler. 1993. Influence of larval food consumption on the fecundity of the lacewing *Chrysoperla carnea.* Entomol. Exp. Appl. 67: 9–14.

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